

PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : A61K 31/00, 31/185, 31/70	A1	(11) International Publication Number: WO 96/39129 (43) International Publication Date: 12 December 1996 (12.12.96)
(21) International Application Number: PCT/CA96/00374 (22) International Filing Date: 6 June 1996 (06.06.96) (30) Priority Data: 08/471,093 6 June 1995 (06.06.95) US (71) Applicants: QUEEN'S UNIVERSITY AT KINGSTON [CA/CA]; Kingston, Ontario K7L 3N6 (CA). NEUROCHEM, INC. [CA/CA]; Fleming Hall, Queen's University of Kingston, Kingston, Ontario K7N 3N6 (CA). (72) Inventors: KISILEVSKY, Robert; 120 Cairn Grove, Kingston, Ontario K7M 4B9 (CA). SZAREK, Walter; Suite 301, 165 Ontario Street, Kingston, Ontario K7L 2Y6 (CA). WEAVER, Donald; 27 College View Crescent, Kingston, Ontario K7M 7J8 (CA). FRASER, Paul; 611 Windermere Avenue, Toronto, Ontario M6S 3L9 (CA). KONG, Xianqi; 47-202 Van Order Drive, Kingston, Ontario K7M 1B6 (CA). (74) Agent: GRENZOWSKI, Deborah, G.; Scott & Aylen, 1000-60 Queen Street, Ottawa, Ontario K1P 5Y7 (CA).		(81) Designated States: AL, AM, AU, BB, BG, BR, CA, CN, CZ, EE, FI, GE, HU, IS, JP, KG, KP, KR, LK, LR, LT, LV, MD, MG, MK, MN, MX, NO, NZ, PL, RO, SG, SI, SK, TR, TT, UA, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: METHODS OF INCREASING AMYLOID DEPOSITION (57) Abstract <i>In vivo and in vitro methods of increasing amyloid deposition using amyloid-enhancing compounds are described. Methods of forming amyloid fibrils and screening for agents useful in treating amyloidosis are also described. Animals having non-naturally occurring amyloid deposits produced using the amyloid-enhancing compounds even further are described.</i>		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgyzstan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	KZ	Kazakhstan	SG	Singapore
CH	Switzerland	LI	Liechtenstein	SI	Slovenia
CI	Côte d'Ivoire	LK	Sri Lanka	SK	Slovakia
CM	Cameroon	LR	Liberia	SN	Senegal
CN	China	LT	Lithuania	SZ	Swaziland
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	LV	Latvia	TG	Togo
DE	Germany	MC	Monaco	TJ	Tajikistan
DK	Denmark	MD	Republic of Moldova	TT	Trinidad and Tobago
EE	Estonia	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	UG	Uganda
FI	Finland	MN	Mongolia	US	United States of America
FR	France	MR	Mauritania	UZ	Uzbekistan
GA	Gabon			VN	Viet Nam

METHODS OF INCREASING AMYLOID DEPOSITION

Related Applications

This application is a continuation-in-part of application Serial No. 08/471,093, filed June 6, 1995, the contents of which is incorporated herein by reference.

Background of the Invention

Amyloidosis refers to a pathological condition characterized by the presence of amyloid. Amyloid is a generic term referring to a group of diverse but specific extracellular protein deposits which are seen in a number of different diseases. Though diverse in their occurrence, all amyloid deposits have common morphologic properties, stain with specific dyes (e.g., Congo red), and have a characteristic red-green birefringent appearance in polarized light after staining. They also share common ultrastructural features and common x-ray diffraction and infrared spectra.

Amyloid can be classified according to the protein type, as described in a recent report (Kazatchkine et al., (1993) *Bull. WHO* 71:105). Different amyloidoses are also characterized by the type of protein present in the deposit. For example, neurodegenerative diseases such as scrapie, bovine spongiform encephalitis, Creutzfeldt-Jakob disease and the like are characterized by the appearance and accumulation of a protease-resistant form of a prion protein (referred to as A^{Sc} or PrP-27) in the central nervous system. Similarly, Alzheimer's disease, another neurodegenerative disorder, is characterized by congophilic angiopathy, neuritic plaques and neurofibrillary tangles, all of which have the characteristics of amyloids. In this case, the plaques and blood vessel amyloid is formed by the beta protein. In other systemic diseases such as adult-onset diabetes, the protein is amylin, in complications of long-term hemodialysis, the protein is beta-2-microglobulin, in sequelae of long-standing inflammation, the protein is serum amyloid A (SAA), and in plasma cell dyscrasias, amyloids are characterized by the accumulation of light chains systemically. In each of these cases, a different amyloidogenic protein is involved in amyloid deposition.

Once these amyloids have formed there is no generally accepted therapy or treatment to dissolve the deposits *in situ*. There is, therefore, an urgent need for therapeutic agents which can either inhibit the formation or growth of amyloid or dissolve amyloid deposits once formed. Co-pending U.S. patent application 08/403,230 describes compositions of sulfated and sulfonated compounds, or functional equivalents thereof, useful in the treatment of amyloidosis *in vivo*, and methods of use thereof.

One difficulty encountered in identification of therapeutic agents for the treatment of amyloidosis is the lack of rapid, general screening assays for such agents. U.S. patent 5,164,295 describes a screening assay which measures the ability of a compound to inhibit the interaction between an amyloid protein and an extracellular matrix protein. U.S. patent 5,348,963 describes an assay which screens compounds based on their ability to alter the intracellular processing of amyloid precursor proteins in mammalian cells in cell culture. Co-pending U.S. patent application 08/403,230 describes a mouse model of amyloidosis which is used for screening for compounds which have the ability to decrease amyloid formation *in vivo*.

Another difficulty encountered in searching for treatments for amyloidosis is that the mechanism of amyloid fibril formation, although extensively studied *in vivo* (see, e.g., R. Kisilevsky and I. Young (1994) in G. Husby, ed. "Clinical Rheumatology: Vol. 8, No. 3, Reactive Amyloidosis and the Acute Phase Response" London: Bailliere Tindall, pp. 613-626; E. Gruys and F.W.J.J. Snel (1994) in Husby, ed., *op. cit.*, pp.599-611) and *in vitro* (see, e.g., G.G. Glenner *et al.*, (1971) *Science* 174:712; E.M. Castano *et al.* (1986) *Biochem. Biophys. Res. Commun.* 141:782; D.A. Kirschner *et al.* (1987) *Proc Natl. Acad. Sci. USA* 84:6953), has not been completely elucidated. In general, *in vitro* conditions used to cause amyloid fibril formation have not been similar to physiological conditions. The *in vitro* methods known in the art usually require high amyloid protein concentrations, unnatural pH ranges, or strong solvents to cause amyloid fibril formation (see, e.g., I.B. Kingston *et al.*, (1995) *Nature Med.*, 1:138-142; Glenner *et al.*, (1971) *op. cit.*). Thus, the amyloid fibrils formed by these methods may not reflect the structure of fibrils formed *in vivo*, and the conclusions drawn from structural studies of such *in vitro*-formed fibrils may not be applicable to fibrils formed by amyloidogenic conditions *in vivo*.

Summary of the Invention

This invention is based, at least in part, on the discovery of amyloid-enhancing compounds which are capable of increasing the rate of amyloid formation both *in vivo* and *in vitro*. The invention pertains to methods useful in increasing amyloid formation in an animal. The methods of the invention find use in animal models of amyloidosis because the induction time or formation time of amyloid formation in the animal models can be decreased . The invention also pertains to methods of inducing amyloid fibril formation *in vitro*.

In one aspect, the invention features a method of increasing amyloid deposition in a mammal. The method comprises administering to the mammal an effective amount of an amyloid-enhancing compound. In preferred embodiments, the amyloid-enhancing compound is a sulfonated aliphatic or aromatic compound. In a

more preferred embodiment, the amyloid-inducing compound has an aromatic moiety comprising at least two sulfonate moieties. Preferred amyloid-enhancing compounds include 4,5-dihydroxy-1,3-benzenedisulfonic acid, 2,5-dihydroxy-1,4-benzenedisulfonic acid, 2,5-dimethoxy-1,4-benzenedisulfonic acid, and their salts, including pharmaceutically acceptable salts. The compounds of the invention are administered to a subject by a route which is effective for enhancement of amyloid deposition. Suitable routes of administration include subcutaneous, intravenous and intraperitoneal injection. The compounds of the invention have been found to be effective at enhancing amyloid deposition when administered orally. Accordingly, a preferred route of administration is oral administration. The compounds can be administered with a pharmaceutically acceptable vehicle.

In another aspect, the invention features a method of forming amyloid fibrils from an amyloidogenic peptide. The method features contacting an amyloidogenic peptide with an effective amount of an amyloid-enhancing compound forming amyloid fibrils.

In still another aspect the invention features a method of screening for agents useful for treating amyloidosis. The method comprises providing a reaction mixture which includes a solution of an amyloidogenic peptide, an amyloid-enhancing compound, and an agent potentially useful for treating amyloidosis, under conditions such that, in the absence of the agent potentially useful for treating amyloidosis, amyloid fibrils would form, and observing formation or absence of amyloid fibrils.

In yet another aspect, the invention provides an animal having non-naturally occurring amyloid deposits. The animal is treated with an effective amount of an amyloid-enhancing compound such that the animal has non-naturally occurring amyloid deposits.

Brief Description of the Drawings

Figure 1 shows circular dichroism (CD) spectra of A β in the absence and presence of WAS-20 (2,5-dihydroxy-1,4-benzenedisulfonic acid).

30

Figure 2 shows circular dichroism (CD) spectra of A β in the absence and presence of WAS-21 (4,5-dihydroxy-1,3-benzenedisulfonic acid).

Detailed Description

This invention pertains to methods useful in increasing amyloid formation in an animal. These methods of the invention find use in animal models of amyloidosis. The invention also pertains to methods of inducing amyloid fibril formation *in vitro*.

35

The *in vitro* methods of the invention find application in the study of amyloidogenesis and amyloid fibril structure.

For convenience, certain terms used throughout the specification and appended claims are first defined.

5 The term "amyloid-enhancing compound", as used herein, means a compound which is capable of enhancing the rate or amount of amyloid deposition *in vivo* or *in vitro*. A compound can be tested to determine whether it is an amyloid-enhancing compound according to the methods described in the Exemplification, *infra*. Preferred amyloid-enhancing compounds for use *in vivo* do not exhibit significant
10 toxicity when administered to an animal at levels sufficient to increase amyloid deposition *in vivo*. Preferred amyloid-enhancing compounds for use *in vivo*, when tested in the mouse model described in Example 1, *infra* (hereinafter referred to as mouse amyloid deposition assay (MADA)), increase the rate or amount of amyloid deposited in mouse spleen by at least about 10 percent, more preferably at least about
15 20 percent, and still more preferably at least about 30 percent compared to control. It will be understood that increases in amyloid deposition can occur at certain dosages or concentrations of an amyloid-enhancing compound, but not at other concentrations or dosages. In light of the teaching herein (see, e.g., Example 1), the skilled artisan will be able to determine suitable dosages or concentrations with no more than routine
20 experimentation. Preferred amyloid-enhancing compounds include compounds which have an aromatic moiety and at least two anionic moieties, e.g. attached to the aromatic moiety. Preferred amyloid enhancing compounds include 4,5-dihydroxy-1,3-benzenedisulfonic acid, 2,5-dihydroxy-1,4-benzenedisulfonic acid, 2,5-dimethoxy-1,4-benzenedisulfonic acid, and their salts, preferably their
25 pharmaceutically acceptable salts.

The terms "amyloidogenic peptide" or "amyloidogenic protein", as used herein, refer to peptides which are capable of forming amyloid deposits. Amyloids have been categorized by the type of amyloidogenic protein contained within the amyloid. Non-limiting examples of amyloids, and their amyloidogenic proteins, are
30 as follows (with the associated disease in parentheses after the amyloidogenic protein): β -amyloid (A β) (Alzheimer's disease, Down's syndrome, hereditary cerebral hemorrhage amyloidosis [Dutch]); amyloid A (AA) (reactive [secondary] amyloidosis, familial Mediterranean Fever, familial amyloid nephropathy with urticaria and deafness [Muckle-Wells syndrome]); amyloid κ L-chain or amyloid λ L-chain (idiopathic [primary], myeloma or macroglobulinemia-associated); A β 2M
35 (chronic hemodialysis); transthyretin amyloid (TTR) (familial amyloid polyneuropathy [Portuguese, Japanese, Swedish], familial amyloid cardiomyopathy [Danish], isolated cardiac amyloid, systemic senile amyloidosis); AIAPP or amylin

(adult onset diabetes, insulinoma); atrial natriuretic factor (isolated atrial amyloid);
procalcitonin (medullary carcinoma of the thyroid); gelsolin (familial amyloidosis
[Finnish]); cystatin C (hereditary cerebral hemorrhage with amyloidosis [Icelandic]);
AApoA-I (familial amyloidotic polyneuropathy [Iowa]); AApoA-II (accelerated
5 senescence in mice); fibrinogen-associated amyloid; lysozyme-associated amyloid;
and ASc or PrP-27 (Scrapie, Creutzfeldt-Jakob disease, Gerstmann-Straussler-
Scheinker syndrome, bovine spongiform encephalitis). It will be understood that
other natural or non-natural proteins or peptides which are capable of forming
amyloid or amyloid-like fibrils *in vivo* or *in vitro* are also considered to be
10 amyloidogenic peptides for the purposes of the present invention. For example,
certain fragments of amyloidogenic peptides can form amyloid fibrils (see, e.g., P.D.
Gorevic *et al.*, (1986) *Biochem. Biophys. Res. Commun.* 147:854) and thus are
considered to be amyloidogenic peptides for the purposes of the present invention.

The term "an animal having non-naturally occurring amyloid deposits", as
15 used herein, refers to an animal which has deposits of amyloid which do not normally
occur in that animal under natural conditions, and which are in excess of the amyloid
deposits which occur when the animal is treated with an inflammatory stimulus and
amyloid enhancing factor (AEF) (see, e.g. R. Kisilevsky and L. Boudreau, (1983)
Lab. Invest., 48, 53-59). For example, Swiss white mice do not normally suffer from
20 amyloid deposits, but Swiss white mice treated with the amyloid-enhancing
compounds of the present invention have enhanced amyloid deposition compared to
control animals (see Example 1).

The term "anionic group", as used herein, refers to a moiety which has a net
negative charge. In preferred embodiments, the anionic group has a negative charge at
25 physiological pH. Exemplary anionic groups include sulfonate, sulfate, carboxylate,
phosphate, phosphonate, and the like. Preferred anionic groups include carboxylate
and sulfur containing moieties (e.g., sulfonate, sulfate and the like).

In one aspect, the invention features a method for increasing amyloid
deposition in an animal. Animals which exhibit amyloidosis are useful in screening
30 assays for compounds which inhibit amyloidosis *in vivo*, as described in R. Kisilevsky
et al., (1995) *Nature Med.* 1:143-148, and in copending U.S. Patent Application No.
08/403,230, the disclosures of both of which are hereby incorporated by reference.
Amyloidosis is induced in mice by treating the animal with an inflammatory stimulus,
in combination with amyloid enhancing factor (AEF), as described in Kisilevsky and
35 Boudreau, (1983) *op. cit.* (see Example 1). To induce long-lasting amyloidosis,
repeated injections of the inflammatory stimulus are often required. Furthermore, in
some animal models of amyloidosis, long periods of time must elapse between

administration of an amyloid-inducing stimulus and the deposition of amyloid (Kisilevsky and Boudreau, *ibid.*).

The methods of the present invention make possible the development of amyloid deposits in animals in a shorter period of time and/or result in an increase in amyloid deposits over a selected period of time when compared to a control. This makes possible more rapid screening of compounds in animal models of amyloidosis (for example, the mouse model described herein). Such rapid screening is particularly desirable when large numbers of candidate compounds are to be tested, as, for example, when structure-activity relationships are being probed, or when searching for compounds for further modification. Moreover, the increased amount of amyloid deposition seen in animals treated with amyloid-enhancing compounds may result in increased sensitivity of assays for compounds effective in the treatment of amyloidosis. Compounds which have a relatively modest effect on amyloid deposition *in vivo* may nevertheless be useful therapeutically, or may provide important structure-activity information.

The mechanism by which the amyloid-enhancing compounds described herein increase amyloid deposition *in vivo* is not precisely known. It is known, however, that sulfated and sulfonated compounds effective for the treatment of amyloidosis *in vivo* (i.e., compounds that decrease amyloid deposition) inhibit an interaction between an amyloidogenic protein and a constituent of basement membrane, and thereby inhibit amyloid deposition (see, e.g., U.S. Patent Application No. 08/403,230 and references cited therein). The constituent of basement membrane is a glycoprotein or proteoglycan, preferably heparan sulfate proteoglycan (HSPG). Without being bound by theory, it is believed that the amyloid-enhancing compounds described herein enhance interactions either between an amyloidogenic protein and a component of basement membrane, or between monomers of the amyloidogenic protein, and thereby enhance amyloid deposition *in vivo*.

The methods of the invention also provide increased amyloid deposition *in vitro*. The mechanism by which the amyloid-enhancing compounds described herein increase amyloid deposition *in vitro* is also not precisely known.

The above-described mouse model of amyloidosis is a useful model of amyloidosis caused by serum amyloid A (SAA). The present invention makes possible the development of animal models of amyloidosis caused by other amyloidogenic proteins and for which no animal models currently exist.

In another embodiment, the invention features a method of increasing amyloid deposition *in vitro*. The *in vitro* study of amyloid fibril formation is important to the ultimate elucidation of the causes of amyloid formation *in vivo*. Co-pending U.S. patent application Serial no. 08/372,178 (the disclosure of which is hereby

incorporated by reference) discloses assay methods useful for studying the effects of test compounds on amyloid deposition *in vitro*. However, most previous studies of *in vitro* amyloid fibril formation have used conditions which are not similar to conditions found *in vivo*. For example, amyloid formation *in vitro* has been induced
5 by use of concentrations of amyloidogenic proteins which are orders of magnitude greater than are found *in vivo*. As noted above, non-physiological pH ranges and organic solvents have also been employed to cause amyloid fibril formation. Furthermore, certain amyloidogenic proteins are resistant to fibrillogenesis *in vitro*, and the study of these resistant amyloidogenic proteins has heretofore been hindered
10 by difficulties encountered in inducing fibril formation.

The use of non-physiological conditions may lead to misinterpretations. The nature of the interactions between HSPG and amyloidogenic proteins is not completely understood. Crystallographic studies of amyloid fibrils formed *in vitro* are a potentially powerful method of studying these interactions; however, the
15 structure of the fibrils may be affected by the conditions used for fibril formation. For example, the structure of amyloid fibrils deposited from a solution at pH 5.6 (the pI of amyloid beta protein fragment 1-28) may not be the same as the structure of fibrils deposited at pH 7. Fibrils deposited from protein solutions of differing concentration may also not be identical. The concentration of amyloidogenic peptides found *in*
20 *vivo*, such as serum amyloid A (SAA), varies, but may be as low as 1-5 µg/ml, under normal circumstances, to as high as about 0.5-1 mg/ml during an inflammatory reaction. For these reasons, conditions for fibril formation which more closely resemble conditions found *in vivo* are desirable.

The present invention provides a method of inducing amyloid fibril formation
25 *in vitro* under conditions which more closely approximate physiological conditions (see Example 2). The structure of the fibrils formed *in vitro* by the methods provided herein is believed to more closely resemble the structure of amyloid fibrils found in subjects suffering from amyloidosis. Thus, in a preferred embodiment, the method of inducing amyloid fibril formation *in vitro* according to the present invention is carried
30 out at a pH between about 6.5 and about 7.5, more preferably at a pH between about 6.2 and 7.2, and most preferably at a pH of about 7. In a preferred embodiment, the method of inducing amyloid fibril formation *in vitro* according to the present invention is carried in a solution which is substantially free of non-aqueous solvents. In a preferred embodiment, the method of inducing amyloid fibril formation *in vitro*
35 according to the present invention is carried out on a solution of amyloidogenic peptide which has a concentration of less than about 2 mg/ml, more preferably less than about 1.5 mg/ml, and still more preferably at a concentration less than about 1 mg/ml.

An advantage of the methods according to the present invention is that the formation of amyloid fibrils is more rapid in the presence of an amyloid-enhancing compound than in the absence of the amyloid-enhancing compound. As described in Example 2, amyloid fibril formation can be a slow process, requiring up to three days.

5 The subject methods increase the speed of amyloid formation and decrease the time necessary for fibril formation.

Interestingly, the amyloid-enhancing compounds described in Examples 1 and 2 share certain structural features in common with compounds known to decrease amyloid deposition *in vivo* (i.e., at least one anionic sulfonate group, see Example 1).

10 Furthermore, in certain embodiments, the amyloid-enhancing compounds comprise at least one aromatic ring, with a sulfonate directly bonded to the ring. Without being bound by theory, the presence of at least one, and more preferably two, anionic moieties on an aromatic ring may be important for amyloid-enhancing activity. However, the amyloid-enhancing compounds of the invention are not limited to
15 compounds comprising aromatic ring structures or anionic moieties.

In another aspect, the invention provides a method of screening for agents useful for treating amyloidosis. In this screening method, a combination of an amyloidogenic peptide and an amyloid-enhancing compound is provided under conditions which allow amyloid fibrils to form; a test agent is introduced into the
20 combination forming a screening medium; and the presence or absence of amyloid fibril formation within the screening medium is detected as an indication of the ability of the test agent to treat amyloidosis. The ability of a compound to prevent amyloid deposition *in vitro* is related to the ability of that compound to prevent amyloid deposition *in vivo* (see e.g., Examples 1-3, *infra*).

25 In another aspect, the invention provides an animal or mammal having non-naturally occurring amyloid deposits, comprising an animal treated with an amyloid-enhancing compound such that the animal has non-naturally occurring amyloid deposits. Animals having non-naturally occurring amyloid deposits are useful as animal models of amyloidosis, and as such are valuable for screening compounds
30 which are useful for treating amyloidosis. Examples of animals or mammals include mice, rats, dogs, cats, pigs, horses, primates and the like.

The invention is further illustrated by the following examples, which should not be construed as further limiting the subject invention. All references, issued patents, and patent applications cited throughout this application are incorporated
35 herein by reference.

Exemplification

AB is a protein associated with Alzheimer's disease. AB peptide (residues 1-40) was prepared and purified as described in Fraser, P.E. et al., *Biochemistry* 31, 10716 (1992). Briefly, the peptide was synthesized by standard solid-phase techniques and purified by HPLC according to well known procedures. This peptide was used for all *in vitro* experiments described below.

The sulfated and sulfonated compounds used in the examples below are commercially available (e.g. Sigma Chemical Co., St. Louis, MO, or Aldrich Chemical Co., Milwaukee, WI) and/or can be synthesized by standard techniques known in the art (see, e.g., Stone, G.C.H. (1936) *J. Am. Chem. Soc.*, 58:488). The compound designated NC551 was synthesized by a three-step procedure (selective hydroxyl group protection, followed by sulfation of the remaining free hydroxyl groups, and removal of the protecting groups) from D-mannitol. The compound NC553 was produced by a one-step reductive cleavage of Congo Red. The compound NC566 was produced by sulfation of methyl lactoside, and NC709 was produced by persulfation of 1,3,5-benzenetriol (available from Aldrich).

Example 1

Swiss white mice weighing 25-30 g were given Amyloid Enhancing Factor (AEF) and AgNO₃ as described previously (Kisilevsky, R. and Boudreau, L. (1983) "The kinetics of amyloid deposition: I. The effect of amyloid enhancing factor and splenectomy" *Lab. Invest.*, 48, 53-59), to induce amyloidosis. Twenty four (24) hours later they were divided into groups. One group served as a control and was maintained on standard laboratory mouse chow and tap water *ad lib*. The test groups received standard chow but their water contained 20 or 50 mM of one of the compounds listed in Table 1, below. One compound, taurine, was tested at concentrations of 5 mM, 10 mM, 20 mM, and 50 mM. All compounds were dissolved in water containing 1.0% sucrose, and the pH adjusted to 7. Water intake was approximately equivalent for all groups. All animals were sacrificed on day six (6) of the experiment, their spleens collected, prepared for sectioning, spleen sections stained with Congo red (Puchtler, H., et al. (1983) "Application of Thiazole Dyes to Amyloid under Conditions of Direct Cotton Dyeing: Correlation of Histochemical and Chemical Data" *Histochemistry*, 77, 431-445), and the percent area occupied by amyloid assessed by an image analysis apparatus and program (MCID M2, Imaging Research Inc., Brock University, St. Catharines, Ontario, Canada).

The results are summarized in Table 1, below.

Table 1

Effect of Sulfated and Sulfonated Compounds
on AA Amyloid Deposition *In vivo* in Mouse Spleen

5

Compound	Concentration (mM)	Amyloid Deposition*	Standard Error
1,5-Pentanedisulfonate†	50	76	11
	20	60	20
1,6-Hexanedisulfonate†	50	117	17
	20	98	26
1,2-Ethanediol disulfate†	50	8	2
	20	36	10
1,3-Propanediol disulfate†	50	11	4
	20	32	11
1,4-Butanediol disulfate†	50	54	22
	20	44	11
Taurine	50	68	15
	20	45	23
	10	34	16
	5	95	33
WAS-10	50	79	22
	20	80	23
WAS-11 (Methyl 5-deoxy-2,3-O-isopropylidene-β-D-ribofuranoside-5-sulfonic acid, sodium salt)	50	114	
	20	114	
WAS-12	50	55	
	20	74	
WAS-13	50	81	
	20	63	

-11-

WAS-14 (4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid, sodium salt)	50	135	27
	20	83	28
WAS-15	50	56	13
	20	102	24
WAS-16	50	48	12
	20	98	30
WAS-17	50	60	21
	20	54	31
WAS-18 (Bis(4-sulfobutyl) ether disodium salt)	50	110	35
	20	97	50
WAS-19	50	61	13
	20	117	28
WAS-20	50	192	37
	20	119	19
WAS-21	50	158	19
	20	130	28
WAS-22 (Sodium (\pm)-10-camphorsulfonate)	50	83	19
	20	155	28
WAS-23	50	66	12
	20	94	11
WAS-24 (Glycerol trisulfate, trisodium salt)	50	103	19
	20	110	15
WAS-27	50	100	18
	20	86	30
WAS-28	50	56	
	20	53	
WAS-34	50	53	
	20	59	
WAS-35	50	51	
WAS-36	50	71	

SUBSTITUTE SHEET

WAS-37	50	100	
	20	102	
WAS-38	50	81	

†As the sodium salt.

*Amyloid deposition is given as a percentage of untreated control. All measurements
5 are the average of 3-5 animals.

The results indicate that animals treated with sodium 1,2-ethanediol
disulfate or sodium 1,3-propanediol disulfate had at least about a 65% decrease in
10 amyloid deposition at 20 mM and at least about a 90% decrease in amyloid
deposition at 50 mM. Animals treated with sodium 1,4-butanediol disulfate (50
mM), sodium 1,5-pentanedisulfonate ((50 mM), taurine (sodium 2-amino-
ethanesulfonate) (10-20 mM), 3-(cyclohexylamino)-1-propanesulfonate (WAS-12)
(50mM), 4-(2-hydroxyethyl)-1-piperazineethanesulfonate (WAS-13) (20mM), 3-
15 (N-morpholino)propanesulfonic acid (MOPS) (WAS-15) or its sodium salt (WAS-
16) (50 mM), sodium tetrahydro-3,4-thiophene-1,1-dioxide-3,4-disulfonate
trihydrate (WAS-19), sodium 4-hydroxybutane-1-sulfonate (WAS-17) (50 mM),
sodium 1,3,5-pentanetriol trisulfate (WAS-28) (20 and 50mM), 2-aminoethyl
hydrogen sulfate (WAS-34) (20 and 50 mM), Indigo Carmine (WAS-35) (50mM)
20 had at least approximately a 40% decrease in amyloid deposition compared to
untreated control animals. Taurine caused decreased amyloid deposition at
concentrations of 10-20 mM, but had less effect at 5 mM or 50 mM.

The results indicate that certain compounds increased the amount of amyloid
deposition *in vivo*. Administration of 4,5-dihydroxy-1,3-benzenedisulfonic acid
25 (WAS-21) or 2,5-dihydroxy-1,4-benzenedisulfonic acid (WAS-20) resulted in
increased levels of splenic amyloid. Without wishing to be bound by theory, it is
believed that, in certain embodiments, at least one, and more preferably two, aromatic
sulfate or sulfonate moieties are important to the amyloid-enhancing activity of the
subject compounds.

30

Example 2

Samples of amyloid were prepared for electron micrographic (EM)
examination as described in Kisilevsky *et al. Nature Med* (1995), *loc. cit.*. Briefly,
negatively-stained fibrils of A β were prepared by floating pioloform carbon-coated

5 grids on peptide solutions (1-2 mg/ml, 20 mM Tris-HCl, pH 7) that had been preincubated for 72 hours at 37°C to ensure extensive A β polymerization. A control sample contained no test compound. Test compounds were added at ten-fold molar excess to preformed amyloid fibrils with a subsequent incubation for 24 hours at 37°C. After the grids were blotted and air-dried, the samples were stained with 1% phosphotungstic acid (w/v) and visualized on a Hitachi H-7000 electron microscope operated at 75 kV. Fibril dimensions were calibrated using tropomyosin paracrystals.

10 A β fibrils were generated and then treated with WAS-6 (1,3-propanediol disulfate, sodium salt). The fibrils treated with WAS-6 showed a marked reduction in numbers, attenuation in length, and had a much coarser appearance than the control fibrils. Thus, WAS-6 can be classified as an amyloid inhibitor *in vitro*.

15 Amyloid fibrils were then generated in the presence of WAS-20 (2,5-dihydroxy-1,4-benzenedisulfonic acid). EM examination of the fibrils formed showed increased amounts of fine fibrils, compared to the control sample. Thus, WAS-20 can be classified as an amyloid enhancer *in vitro*. In a similar experiment, WAS-21 was classified as an amyloid enhancer *in vitro*. These results are consistent with the *in vivo* assay performed in Example 1, *supra*. WAS-46 (2,5-dimethoxy-1,4-benzenedisulfonic acid), a methylated derivative of WAS-20, was also found to be an amyloid enhancer *in vitro*. This result demonstrates that a free phenolic hydroxyl group is not necessary for amyloid enhancing activity.

20 Amyloid fibrils were then generated and treated with WAS-52 (4-amino-3-hydroxy-1-naphthalenesulfonic acid) or WAS-53 (3,4-diamino-1-naphthalenesulfonic acid). EM examination of the fibrils formed showed no increase in number of fibrils compared to a control sample. Thus, WAS-52 and WAS-53 do not appear to be amyloid enhancers by this *in vitro* assay. In certain *in vitro* assays, however, these compounds do appear to have amyloid-enhancing properties (see, e.g., Example 5). Thus, WAS-52 and WAS-53 do have an affect on amyloid fibril formation and will have utility as either enhancers or inhibitors of amyloid deposition.

30 Example 3

β -pleated sheet secondary structure is characteristic of all amyloid deposits, so destabilization of β -sheet formation may reduce the rate of amyloid deposition; conversely, compounds which increase β -sheet formation may increase the rate of amyloid deposition. Determination of amyloid protein conformation by CD has been reported (McCubbin, W.D. et al., *Biochem J.* 256, 775 (1988)); the amount of β -sheet present in a sample was related to the minimum at about 218 nm.

All CD experiments were performed on a Jasco J-720 instrument. The cell was maintained at 25°C using a circulating water bath. Computer-averaging of traces was performed to improve signal-to-noise ratios. The solvent signal was subtracted.

CD experiments were performed for each test compound according to the following procedure:

A stock solution of purified peptide was made by dissolving the peptide in phosphate-buffered saline (PBS) to a concentration of 2 mg/ml. A test solution was made for each potential therapeutic agent (test compound) as shown below:

	A β stock solution	25 μ l
10	Test compound (20 mg/ml)	2.5 μ l
	Distilled water	2.5 μ l
	10 mM Tris buffer, pH 7	370 μ l

The control sample had no test compound, and a total of 5 μ l distilled water was added. The test solution was incubated for either 0 or 24 hours at 37°C before CD measurement.

Figure 1 shows the CD spectrum of A β in the presence and the absence of compound WAS-20 (2,5-dihydroxy-1,4-benzenedisulfonic acid). The spectra show the effect of WAS-20 on the secondary structure of the amyloid peptide. The size of the minimum near 218 nm is diagnostic of the presence of β -pleated sheet. The amount of β -pleated sheet by this measurement is about 20% greater in the presence of WAS-20; as previously noted, compounds which increase β -sheet formation may increase the rate of amyloid deposition. WAS-20 also increases the amount of light-scattering from the sample, as evidenced by the greatly increased noise at shorter wavelengths; this scattering is believed to be the result of larger aggregated particles produced by the test compound. Thus, WAS-20 can be classified as an amyloid enhancer by this *in vitro* assay. Similar results were obtained with WAS-21 (4,5-dihydroxy-1,3-benzenedisulfonic acid), as shown in Figure 2. Although the change in the minimum near 218 nm is not as large for WAS-21 as for WAS-20, the increased light-scattering due to larger aggregated particles is evident. These results are consistent with the *in vivo* and *in vitro* assays performed in Examples 1 and 2, *supra*.

Example 4

In light of the *in vivo* and *in vitro* amyloid-enhancing activity of WAS-20 and WAS-21, as described above, the effects of additional compounds were assessed in mice using the method of Example 1, *supra*. The results of certain of these tests are shown below in Table 2.

Table 2

Compound	Concentration (mM)	Amyloid Deposition
WAS-46	50	154
	20	62
NC551	32	173, 98 ^a
	13	146, 64 ^a
WAS-52	52	103
	21	123
WAS-53	53	132, 68 ^b
	21	162, 79 ^b

^aResults of two experiments: the first numbers of each pair (173, 146) were obtained in one experiment, and the second numbers (98, 64) were obtained in a second experiment.

^bResults of two experiments: the first numbers of each pair (132, 162) were obtained in one experiment, and the second numbers (68, 79) were obtained in a second experiment.

The results shown in Table 2 indicate that WAS-52 (4-amino-3-hydroxy-1-naphthalenesulfonic acid) does not significantly enhance amyloid deposition under these conditions. However, WAS-46 does appear to enhance amyloid deposition, at least at certain concentrations, which is consistent with the *in vitro* data for WAS-46 described in Example 2.

Although the results of two trials are not entirely consistent for NC551 (D-Mannitol-2,5,-disulfate disodium salt), it is believed that NC551 is an enhancer of amyloid deposition under at least some conditions. In both trials, amyloid deposition was greater at higher doses of the compound (173 vs. 146; 98 vs. 64), suggesting a dose dependence. Also, the average of both trials suggests an enhancement of amyloid deposition at least at the higher dosage level.

The results of the two trials of WAS-53 are also somewhat divergent. However, the first trial indicates that WAS-53 can enhance amyloid deposition *in vivo* under certain conditions.

Example 5

The ability of test compounds to cause amyloid aggregation was measured *in vitro* by direct protein assay. Briefly, test compounds (ten-fold molar excess over the

-16-

peptide, typically about 50 μ mol) were incubated with A β 1-40 peptide, and the reaction mixture was incubated to allow fibrils to form. After incubation, the reaction mixture was centrifuged and the amount of protein remaining in the supernatant was assayed by a spectrophotometric method (Bradford assay, Biorad). The amount of supernatant protein in the absence of a test compound was assigned a value of 100. Test compounds were compared to the control. Compounds which enhance amyloid deposition cause protein to aggregate and thus to be removed from the supernatant by centrifugation. Thus, low supernatant protein concentrations (less than 100) indicate enhancement of amyloid aggregation. In Table 3, below, the results of the assay are shown for several compounds.

Table 3

Compound	Protein Concentration (percent of control)
WAS-20	98
WAS-21	53
NC551	109
WAS-52	18
WAS-53	11
NC566	59
NC709	62

15

The results shown in Table 3 indicate that WAS-52 and WAS-53 are potent enhancers of amyloid deposition in this *in vitro* assay system. The compounds WAS-21, NC566 (methyl beta-D-lactoside heptasulfate, heptasodium salt), and NC709 (1,3,5,-benzenetriol trisulfate, trisodium salt) are also enhancers of amyloid aggregation by this measure. It is further believed that NC566 and NC709 are novel compounds. Thus, NC566 and NC709 do have an affect on amyloid fibril formation and will have utility as either enhancers or inhibitors of amyloid deposition. Accordingly, the invention further contemplates a sulfated compound being methyl beta-D-lactoside heptasulfuric acid or a salt thereof; and a sulfated compound being 1,3,5-benzenetriol trisulfuric acid, or a salt thereof. The invention also includes a composition comprising methyl beta-D-lactoside heptasulfuric acid, or a salt thereof; and a composition comprising 1,3,5-benzenetriol trisulfuric acid, or a salt thereof. The invention further contemplates a pharmaceutical composition comprising methyl beta-D-lactoside heptasulfuric acid, or a pharmaceutically acceptable salt thereof, and

25

a pharmaceutically acceptable vehicle; and the invention also includes a pharmaceutical composition comprising 1,3,5-benzenetriol trisulfuric acid, or a pharmaceutically acceptable salt thereof, and a pharmaceutically acceptable vehicle.

5

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, numerous equivalents to the specific procedures described herein. Such equivalents are considered to be within the scope of this

10 invention and are covered by the following claims.

What is claimed is:

1. A method of increasing amyloid deposition in a mammal, comprising administering to the mammal an effective amount of an amyloid-enhancing compound such that an increase of amyloid deposition occurs in the mammal.
2. The method of claim 1, wherein the amyloid-enhancing compound has an aromatic moiety and at least two anionic moieties.
3. The method of claim 1, wherein the amyloid-enhancing compound enhances amyloid deposition by at least about 10 percent as measured by mouse amyloid deposition assay (MADA).
4. The method of claim 1, wherein the amyloid-enhancing compound is 4,5-dihydroxy-1,3-benzenedisulfonic acid or a pharmaceutically acceptable salt thereof.
5. The method of claim 1, wherein the amyloid-enhancing compound is 2,5-dihydroxy-1,4-benzenedisulfonic acid or a pharmaceutically acceptable salt thereof.
6. A method of forming amyloid fibrils from an amyloidogenic peptide, comprising contacting an amyloidogenic peptide with an effective amount of an amyloid-enhancing compound forming amyloid fibrils.
7. The method of claim 5, wherein the amyloid-enhancing compound has an aromatic moiety and at least two anionic moieties.
8. The method of claim 6, wherein the amyloid-enhancing compound is 2,5-dimethoxy-1,4-benzenedisulfonic acid or a pharmaceutically acceptable salt thereof.
9. The method of claim 6, wherein the amyloid-enhancing compound is 4,5-dihydroxy-1,3-benzenedisulfonic acid or a pharmaceutically acceptable salt thereof.
10. The method of claim 6, wherein the amyloid-enhancing compound is 2,5-dihydroxy-1,4-benzenedisulfonic acid or a pharmaceutically acceptable salt thereof.
11. The method of claim 6, wherein the amyloidogenic peptide is in solution.

12. A method of screening for agents useful for treating amyloidosis, comprising providing a combination of an amyloidogenic peptide and an amyloid-enhancing compound under conditions which allow amyloid fibrils to form; introducing a test agent into the combination forming a screening medium; and detecting the presence or absence of amyloid fibril formation within the screening medium as an indication of the ability of the test agent to treat amyloidosis.
13. The method of claim 12, wherein the amyloid enhancing compound has an aromatic moiety and at least two anionic moieties.
14. The method of claim 12, wherein the amyloid-enhancing compound is 2,5-dimethoxy-1,4-benzenedisulfonic acid or a salt thereof.
15. The method of claim 12, wherein the amyloid-enhancing compound is 4,5-dihydroxy-1,3-benzenedisulfonic acid or a salt thereof.
16. The method of claim 12, wherein the amyloid-enhancing compound is 2,5-dihydroxy-1,4-benzenedisulfonic acid or a salt thereof.
17. An animal having non-naturally occurring amyloid deposits, comprising an animal treated with an effective amount of an amyloid-enhancing compound such that the animal has non-naturally occurring amyloid deposits.
18. The animal of claim 17, wherein the amyloid enhancing compound has an aromatic moiety and at least two anionic moieties.
19. The animal of claim 17, wherein the animal is a mammal.
20. The animal of claim 18, wherein the animal is a mouse.
21. The animal of claim 17, wherein the amyloid-enhancing compound is 2,5-dimethoxy-1,4-benzenedisulfonic acid or a pharmaceutically acceptable salt thereof.
22. The animal of claim 17, wherein the amyloid-enhancing compound is 4,5-dihydroxy-1,3-benzenedisulfonic acid or a pharmaceutically acceptable salt thereof.

23. The animal of claim 17, wherein the amyloid-enhancing compound is 2,5-dihydroxy-1,4-benzenedisulfonic acid or a pharmaceutically acceptable salt thereof.
24. The method of claim 2, wherein at least two anionic moieties are sulfonate moieties.
25. A sulfated compound being methyl beta-D-lactoside heptasulfuric acid, or a salt thereof.
26. The sulfated compound of claim 25, wherein the compound is methyl beta-D-lactoside heptasulfate, heptasodium salt.
27. A sulfated compound being 1,3,5-benzenetriol trisulfuric acid, or a salt thereof.
28. The sulfated compound of claim 27, wherein the compound is 1,3,5-benzenetriol trisulfate, trisodium salt.
29. A composition comprising methyl beta-D-lactoside heptasulfuric acid, or a salt thereof..
30. A composition comprising 1,3,5-benzenetriol trisulfuric acid, or a salt thereof.
31. A pharmaceutical composition comprising methyl beta-D-lactoside heptasulfuric acid, or a pharmaceutically acceptable salt thereof, and a pharmaceutically acceptable vehicle.
32. A pharmaceutical composition comprising 1,3,5-benzenetriol trisulfuric acid, or a pharmaceutically acceptable salt thereof, and a pharmaceutically acceptable vehicle.

1/2

ALZHEIMER A β 1-40 / WAS-20
Incubation for 24 hrs at 37°C

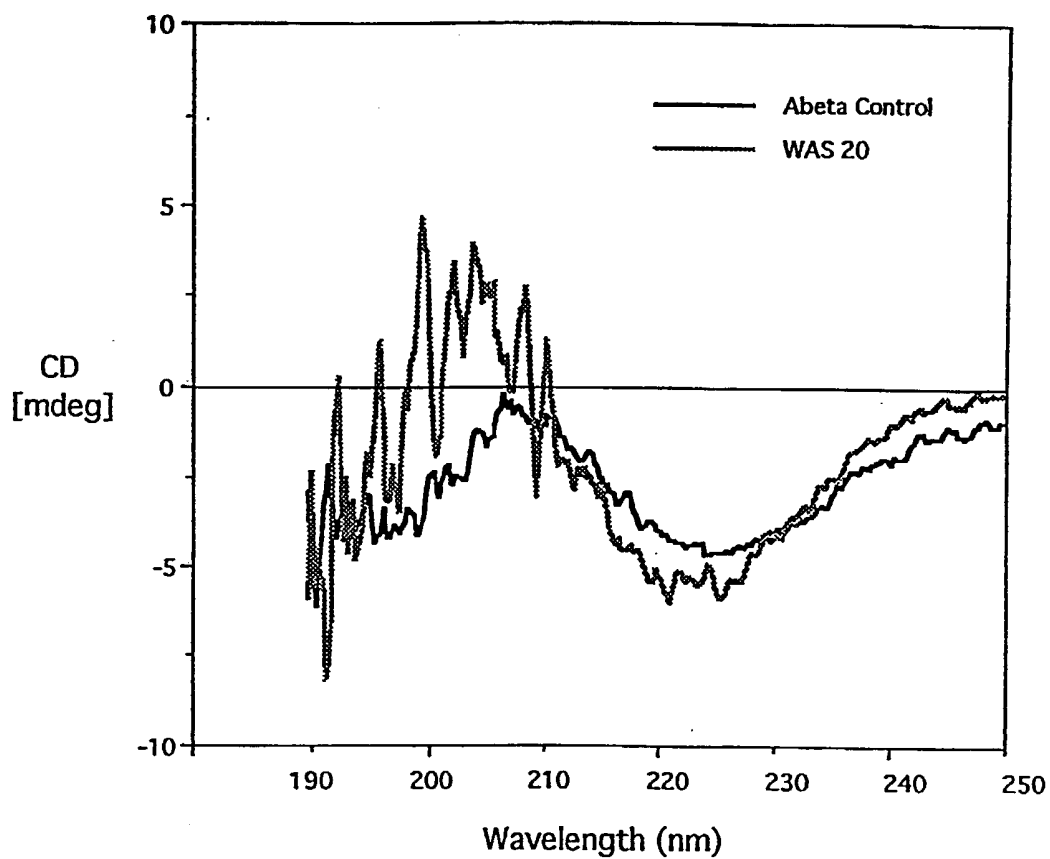


FIGURE 1

SUBSTITUTE SHEET

2/2

ALZHEIMER A β 1-40 / WAS-21
Incubation for 24 hrs at 37°C

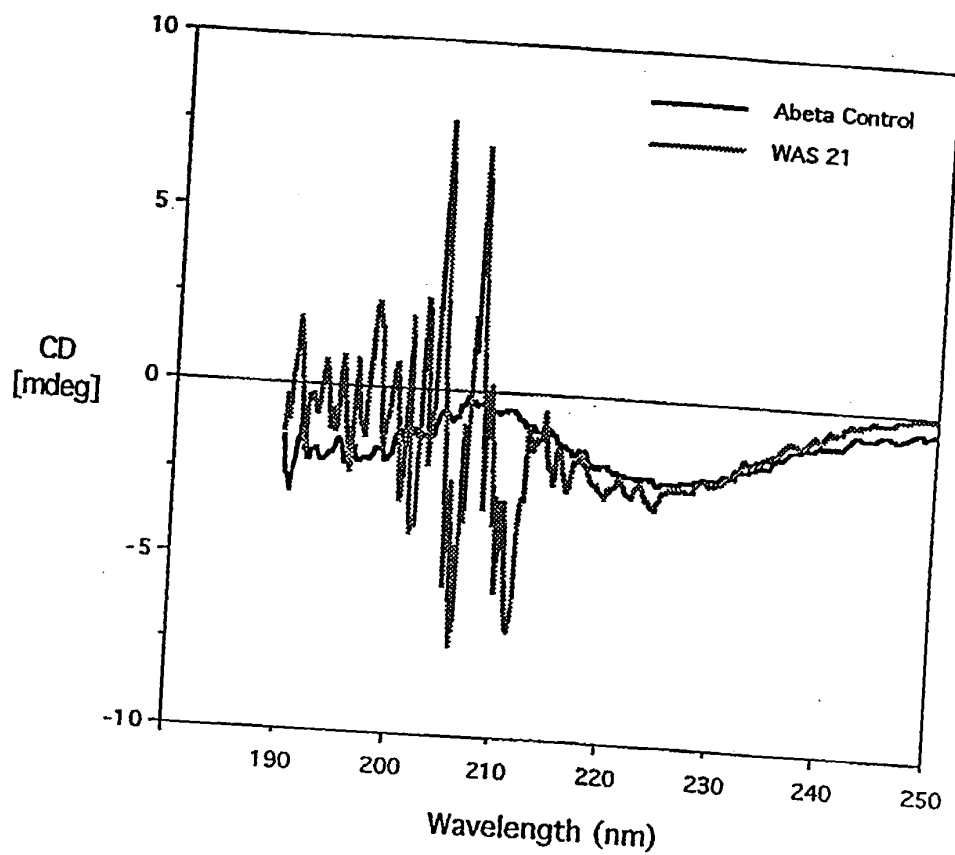


FIGURE 2

SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

Inter: nal Application No

PCT/CA 96/00374

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 A61K31/00 A61K31/185 A61K31/70

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,94 22885 (QUEEN'S UNIVERSITY AT KINGSTON) 13 October 1994 see page 6 see page 9 - page 10; claim 14 ---	25,26, 29,31
X	CAN.J.CHEM., vol. 56, no. 20, 1978, pages 2681-2685, XP000603259 M.A.RAGAN: "Phenol sulfate esters: ultraviolet, infrared, 1H and 13C nuclear magnetic resonance spectroscopic investigation" see page 2685, left-hand column --- -/--	27,28, 30-32

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- "A" document member of the same patent family

Date of the actual completion of the international search

10 October 1996

Date of mailing of the international search report

18. 10. 96

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Theuns, H

INTERNATIONAL SEARCH REPORT

Inter national Application No

PCT/CA 96/00374

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	J.CHROMATOGR., vol. 178, no. 2, 1979, pages 505-513, XP000601854 M.A.RAGAN ET AL.: "PAIRED-ION REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF PHENOL SULFATES IN SYNTHETIC MIXTURES, ALGAL EXTRACTS AND URINE" see page 508 ---	17-28, 30,32
X	BULL. LIAISON-GROUPE POLYPHENOLS, vol. 14, 1988, pages 244-247, XP000603304 K.-W. GLOMBITZA ET AL.: "Phenolsulfate esters from Phaeophyceae" see page 246 ---	27,28, 30,32
A	US,A,5 164 295 (KISILEVSKY ET AL.) 17 November 1992 see the whole document ---	1
A	WO,A,94 22437 (QUEEN'S UNIVERSITY AT KINGSTON) 13 October 1994 see the whole document -----	1

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CA96/00374

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 1-16, 24 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☒ Claims Nos.: 1-3, 6-7, 12-13, 24
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
In view of the definition of the compounds in claims 1-3, 6-7, 12-13 and 24 a complete search is virtually impossible.
Claims not searched: 17-23
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter: val Application No

PCT/CA 96/00374

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9422885	13-10-94	CA-A-	2159326	13-10-94
		CA-A-	2159649	13-10-94
		WO-A-	9422437	13-10-94
		EP-A-	0691844	17-01-96
		EP-A-	0691976	17-01-96
		JP-T-	8508260	03-09-96

US-A-5164295	17-11-92	NONE		

WO-A-9422437	13-10-94	CA-A-	2159326	13-10-94
		CA-A-	2159649	13-10-94
		WO-A-	9422885	13-10-94
		EP-A-	0691844	17-01-96
		EP-A-	0691976	17-01-96
		JP-T-	8508260	03-09-96
